

Supporting Information

O-GlcNAcylation of Core Components of the Translation

Initiation Machinery Regulates Protein Synthesis

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Materials and methods

Cell culture and tumor tissues. Cell lines 293T, IOSE80, A2780, OVCAR-8, OVCAR-10, SKOV-3, GES-1, MGC803, KATO3, HGC27, MCF7, MCF10A, MDA-MB-231, LO2, HepG2, Hep3B were all obtained from ATCC and cultured according to ATCC protocols. Among them, IOSE80, GES-1, MCF10A, and LO2 are considered as noncancerous epithelia cells derived from ovarian, stomach, breast, and liver tissues, respectively. Ovarian tumor tissues and normal tissues, breast tumor tissues and matching tumor-adjacent normal tissues from the same patient were obtained from the Zhejiang Cancer Hospital Bio-specimen Repository (Hangzhou, China). Informed consent was obtained from the patients. Procedures related to human subjects were approved by the Ethic Committee of Zhejiang Cancer Hospital.

Immunoblotting. Cells were lysed in SDS lysis buffer (1% SDS, 50mM Tris-HCl, pH 7.5, 100 mM NaCl, and Complete protease inhibitors (Roche)), and the lysate was resolved on a 4–12% SDS-PAGE gel, transferred to nitrocellulose membrane, and immunoblotted with the indicated antibodies. Antibodies used in this study were obtained from the following sources: anti-O-GlcNAc antibody (RL2, clone18B10.C7, Thermo Scientific, 1:1,000), anti-eIF4AI antibody (Abcam, 1:1,000), anti-eIF4B antibody (clone EP2299Y, Abcam, 1:1,000), anti-eIF4E antibody (clone Y448, Abcam, 1:500), anti-PDCD4 antibody (Sigma-Aldrich, 1:650), anti-eIF4GI antibody (clone D6A6, Cell Signaling technology, 1:1,000), anti-PABP1 antibody (Sigma-Aldrich, 1:1,000), anti-Flag antibody (clone M2, Sigma-Aldrich, 1:5,000), anti-Myc antibody (Abmart, 1:5,000), anti-GAPDH antibody (clone G6, Santa Cruz Biotechnology). Western blots were visualized and quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Version 2.1).

m⁷GDP affinity chromatography. The protein extracts were prepared from 293T cells in the extraction buffer (50 mM HEPES pH 7.5, 150 mM KCl, 1 mM EDTA, 2 mM DTT, and 1% Triton X-100), and incubated with m⁷GDP sepharose (Jena Bioscience) for 2 h at 4°C. The beads were pelleted and washed extensively (5 times) with the extraction buffer. The bound proteins were eluted with the extraction buffer containing m⁷GDP and applied to analysis (1). A control experiment was performed by incubating the same protein extracts with unconjugated protein-G agarose. A duplicate experiment was performed.

Analysis of eIF4AI, eIF4GI, eIF4E, eIF4B and eIF4H glycosylation. Chemoenzymatic labeling and biotinylation of proteins in cell lysates were carried out as described previously (2). Briefly, cell lysate (500 µg) was labeled according to the Click-iT O-GlcNAc Enzymatic Labeling System protocol (Invitrogen), and conjugated with an alkyne-biotin compound as per the Click-iT Protein Analysis Detection Kit protocol (Invitrogen). Control experiments were carried out in parallel in the absence of the labeling enzyme GalT or UDP-GalNAz. Biotinylated lysates were precipitated using methanol and chloroform as described in the Click-iT Protein

Analysis Detection Kit protocol, resolubilized in 1% SDS, and neutralized with an equal volume of neutralization buffer (6% NP-40, 100 mM Na₂HPO₄, 150 mM NaCl). Lysates were then incubated with streptavidin resin (Pierce) with end-to-end rotation at 4°C overnight. Resins were then washed five times with 1 mL of low-salt buffer (100 mM Na₂HPO₄, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate) and five times with 1 mL of high-salt buffer (100 mM Na₂HPO₄, 500 mM NaCl, 0.2% TritonX-100). Biotinylated proteins were eluted by boiling the resin in 50 mM Tris-HCl pH 6.8, 2.5% SDS, 100 mM DTT, 10% glycerol and 20 mM biotin for 10 min. Western blotting analysis was carried out with anti-eIF4AI, anti-eIF4GI, anti-eIF4E, anti-eIF4B, or eIF4H antibodies.

To quantify the level of glycosylation, the intensity of the total eIF4AI or eIF4GI protein band (Input) and the glycosylated eIF4AI or eIF4GI protein band (Elution) were measured, and the ratio of the intensity of the glycosylated protein versus the intensity of the total protein was taken as the level of glycosylation.

Co-immunoprecipitation assay. Cell lysate (500 µg) was incubated with indicated antibodies overnight at 4°C. 100 µL protein A/G resin (Pierce) slurry was mixed with the lysate-antibody mixture and incubated with gentle agitation for 2 h at room temperature. The resin was washed extensively with IP buffer (25 mM Tris-HCl, 150 mM NaCl pH 7.2) and eluted with the elution buffer (0.1 M Glycine-HCl pH 3.0).

Expression and purification of recombinant His-tagged eIF4AI and GST-tagged eIF4GI₂₋₃₀₀. Recombinant eIF4AI was expressed and purified as previously described (3). Briefly, BL21 (DE3) codon+ *E. coli* host cells were transformed with pET28a-His₆-eIF4AI, cultured at 37°C until the OD 600 reached 0.6, and induced with 1 mM IPTG for 3 h. Recombinant His₆-eIF4AI was enriched on a Ni²⁺-NTA agarose column, further purified on a Q-Sepharose fast flow matrix, and eluted with a linear salt gradient (100-500 mM KCl). Fractions containing recombinant His₆-eIF4AI were pooled and dialyzed against the dialysis buffer (20 mM Tris-Cl pH 7.5, 10% glycerol, 0.1 mM EDTA) overnight at 4°C.

Recombinant GST-eIF4GI₂₋₃₀₀ was expressed and purified as described (4). Briefly, BL21 (DE3) codon+ *E. coli* host cells were transformed with pGEX-eIF4GI, and induced expression at OD 600 = 0.5 with 1 mM IPTG for 4 h. GST-eIF4GI was isolated and purified according to the protocol from ThermoFisher Scientific.

Site mapping of eIF4AI glycosylation. Flag-tagged eIF4AI and HA-tagged OGT were co-transfected in 293T cells. After 48 h, Flag-tagged eIF4AI was isolated using anti-Flag M2 agarose beads with gentle agitation for 2 h at room temperature. After extensive washing, the bound protein was eluted in a buffer containing 4% SDS and 100 mM Tris-HCl, pH 8.0. After SDS-PAGE (4–12% Bis-Tris gels) and staining with Bio-Safe Coomassie blue R250 (0.25%) Stain (Bio-Rad), the eIF4AI protein band was excised and manually digested in-gel with trypsin. The extracted peptides were lyophilized and further purified by reverse-phase HPLC (Agilent1100) using a preparative reverse-phase column (Agilent Eclipse XDB-C18; 5 mm, 9.4×250mm)

and a gradient of 5–30% B buffer over 20 min at 4 ml min⁻¹ (A buffer, 0.5% aqueous AcOH; B buffer, 100% MeCN). Fractions eluting between 5–12 min were collected, pooled, lyophilized and subjected to nanoLC-LTQ-CID/ETD-MS analysis on an LTQ-Orbitrap Velos as previously described (5). Data search was performed by Proteome Discovery (MASCOT search engine, version 1.3) with O-GlcNAc (Ser/Thr) set as variable modification.

eIF4AI duplex unwinding activity (helicase) assays. The helicase activity of eIF4AI WT and mutants were measured using a real-time fluorescence assay as described with slight modifications (6). Briefly, fluorescence reporter strand and loading strand of oligonucleotides were chemically synthesized and modified. Sequences of the oligonucleotides used in the assays are shown below:

Cy3-labeled reporter strand 5-Cy3-GCUUUACGGUGC-3

Quenching strand 5-GAACAACAACAACAACCAUGGCACCGUAAAGCBHQ-3

DNA capture strand 5-GCACCGTAAAGC-3

Unwinding reactions were performed in a 96-well RNase free white plate (Thermo) by using an EnVision Multilabel Reader (PerkinElmer). The duplex substrate (reporter strand and quenching strand) was firstly incubated with WT or different mutants of recombinant eIF4AI (1 μ M), and the unwinding reaction was initiated by the addition of 2 mM ATP-Mg²⁺ at 37°C. The increase in fluorescence was calibrated to the fraction of duplex unwound over time. Triplicate experiments were performed.

Generation of eIF4AI rescue A2780 stable cell lines. To generate the eIF4AI rescue A2780 cell lines, a retroviral complementation vector (RCV) system that affords simultaneous small hairpin RNA (shRNA)-mediated depletion of endogenous eIF4AI while co-expressing exogenous Flag-tagged eIF4AI (WT, S322/323A, S322/323Y) was employed as described (7). The shRNA sequence that knockdown the human eIF4AI was cloned to replace the mouse shRNA in the original RCV.

The shRNA sequence:

5'-CCGGCCTTGTATCAAGGGTTATGATCTCGAGATCATAACCCTTGATACA
AGGTTTTTTG-3' (obtained from the Public TRC Portal database)

The corresponding scramble sequence:

5'-CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGG-CGACTTAACC
TTAGGTTTTTTG-3'

The exogenous eIF4AI sequence was made resistant to the eIF4AI shRNA by introducing silent mutations (lower case: 5'- CCtTGtATcAAGGGtTAtGAt -3') using the enzyme PrimeSTAR Max DNA Polymerase (Takara). A2780 cells were infected with the retroviruses and selected for monoclonal cells with green fluorescence.

CRISPR-Cas9-mediated editing of eIF4GI in MCF7 cells. The eIF4GI S61A knock-in MCF-7 cells were generated using EGETM system (Extreme Genome Editing System). Cas9/sgRNA plasmid was designed against exon 2 of the human eIF4GI gene. The sgRNA sequence used is 5'-CGAGCAGTGCAGCCTCCCGA-3'. DNA was introduced into MCF-7 cells by Polyject transfection reagent containing 1

µg of Cas9/sgRNA plasmid and 2 µg of ssODN (GENEWIZ). One day after transfection, puromycin (2 µg /mL) was added to the culture medium, to allow single cell-derived colony formation. Surviving clones were picked and sent for genotyping. Characterization of the eIF4GI locus was determined via PCR amplification of the targeted locus using the following primers:

fwd: 5'-GGTAGAGGTGACGTGTGTATGCA-3',

rev: 5'-CAAGCCAACTCTGGGCAATGA-3'.

Fluorescence anisotropy assay. The assay was performed as previously described (4). Briefly, poly(A)₅₀ RNA with 3'-end labeled with fluorescein were obtained from Thermo. To determine dissociation constant (K_d) for GST-eIF4GI-poly(A)₅₀ complexes, GST-eIF4GI WT, S61A or S61Y was added to poly(A)₅₀-FL in 300 µL of 1X Recon Buffer (30 mM HEPES-KOH pH 7.4, 100 mM KOAc pH 7.6, 3 mM Mg(OAc)₂, 2 mM DTT) in a quartz cuvette. The fluorescence anisotropy of was measured on a Spex Fluorolog-3 spectrofluorometer with an excitation wavelength of 497 nm and emission wavelength of 520 nm. K_d was calculated by fitting the anisotropy data to a hyperbolic function using Kaleidagraph software (Synergy). Triplicate experiments were performed.

In vitro translation assay. In vitro translation assays were performed using the Flexi® Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Briefly, indicated amounts of eIF4A S322/323Y mutant protein (0, 0.5, 1.0, 1.5, 2.0 µg) were added to the reaction mixture (Flexi® Rabbit Reticulocyte Lysate, 10 µM Amino Acid Mixture, RNasin® Ribonuclease Inhibitor, 7 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1mg/mL Luciferase Control RNA, 1 µg eIF4A WT). The translation reaction proceeded at 30°C for 90 minutes and the luciferase signal was recorded on a Victor 3 microplate reader. In the assays with DMSO-treated or TMG-treated eIF4A proteins, 2.0 µg of the eIF4A protein was used in the assay. Triplicate experiments were performed.

Protein crosslinking with a cleavable chemical crosslinker. The crosslinking experiment was performed using Sulfo-SDAD (Sulfo-NHS-SS-Diazirine, Thermo Scientific Pierce). In brief, cells were lysed in low salt lysis buffer (0.5% NP40, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, and complete protease inhibitors), and adjusted to a final concentration of 5 mg/mL in the lysis buffer. The working concentration of Sulfo-SDAD is 1 mM. The mixture was incubated with gentle shaking for 1 h at 4°C. Then, the crosslinking reaction was initiated with long wavelength UV-light (366 nm), and continued for 30 min on ice. The reaction was quenched by adding 50 µL of 1 M Tris-HCl (pH 7.5). Reducing agents (e.g., DTT or 2-mercaptoethanol) were added to de-crosslink the interacting proteins when the subsequent protein analysis was performed.

In vivo translation assay. 1×10⁴ cells were seeded per well in a 12-well plate and were cultured for overnight, and were incubated in methionine-free medium for 30

min prior to the addition of the methionine analog L-homopropargylglycine (HPG, 50 μ M) and continued to culture for another 30 min. After cell fixation and permeabilization with 0.5% Triton X-100, the Click-iT reaction with Alexa-488 detection reagent was performed for 30 min at room temperature. After the Click-iT reaction, cells were subjected to immunofluorescence detection. Fluorescent signal intensity was normalized with cell numbers and represented the amount of newly synthesized protein pools. Triplicate experiments were performed.

Colony formation assay. Five hundred cells were seeded per well in a 12-well plate with 4% soft agar, and were cultured for 14 days. The culture media were changed every 3 days. Wells were washed with PBS and colonies were calculated under microscope. A duplicate experiment was performed.

Polysome analysis. 3×10^7 Cells were washed twice with ice-cold PBS supplemented with 100 μ g/mL cycloheximide and lysed with hypotonic lysis buffer (5 mM Tris-HCl pH 7.5, 2.5mM $MgCl_2$, 1.5 mM KCl, 2 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate, 100 μ g/L cycloheximide). The supernatant was loaded onto a buffer containing 10-50% sucrose gradient and centrifuged at 39000 rpm for 2.5 hr at 4°C. The absorbance of fractionated fractions was recorded at UV 254 nm (Brandel). Triplicate experiments were performed.

Cell cycle synchronization and flow cytometry analysis. 293T cells were cultured in the complete DMEM medium for 24 h, before 2.5 mM thymidine (Sigma) was added. Following the treatment with thymidine for another 15 h, the culture medium was replaced with fresh medium without thymidine. Cells were then collected at 0, 4 h, 6 h and 8 h after medium replacement. Cells were further determined to be at a specific cell cycle stage based on flow cytometry profiling of the DNA content. For cell cycle analysis, cells were trypsinized, washed in PBS three times, and fixed in 70% ethanol at 4°C for at least 2 h. Ethanol was removed by centrifugation at 1000 g for 2 min. Cells were stained with propidium iodide (50 μ g/mL) in the presence of RNase A (50 μ g/mL) in PBS for 30 min at 37°C. DNA content analysis was performed using a NovoCyt flow cytometer.

Cell proliferation analysis. Cell proliferation assays were performed by seeding 5,000 cells per well in a 12-well tissue culture plate, and determined by counting cell numbers as stained by trypan blue every 24 h for 6 days. Triplicate experiments were performed.

Statistical analysis. P values were calculated from Student's paired t-test when comparing within groups and from Student's unpaired t-test when comparing between groups. For those analyses where more than one t-test is applied to the same data set, statistical analysis was performed by one-way analysis of variance and Bonferroni comparison post-test.

References:

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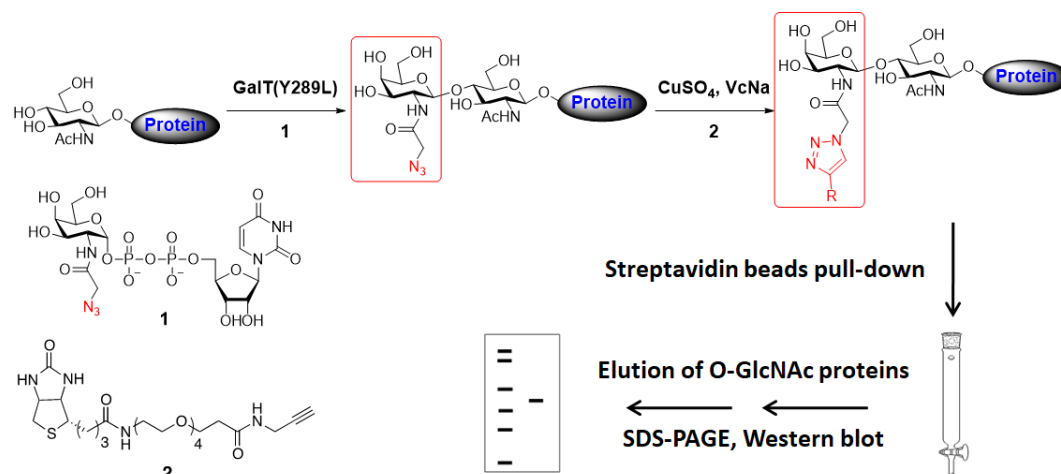


Figure S1. Chemo-enzymatic strategy for selective enriching and detecting O-GlcNAcylated proteins of interest.

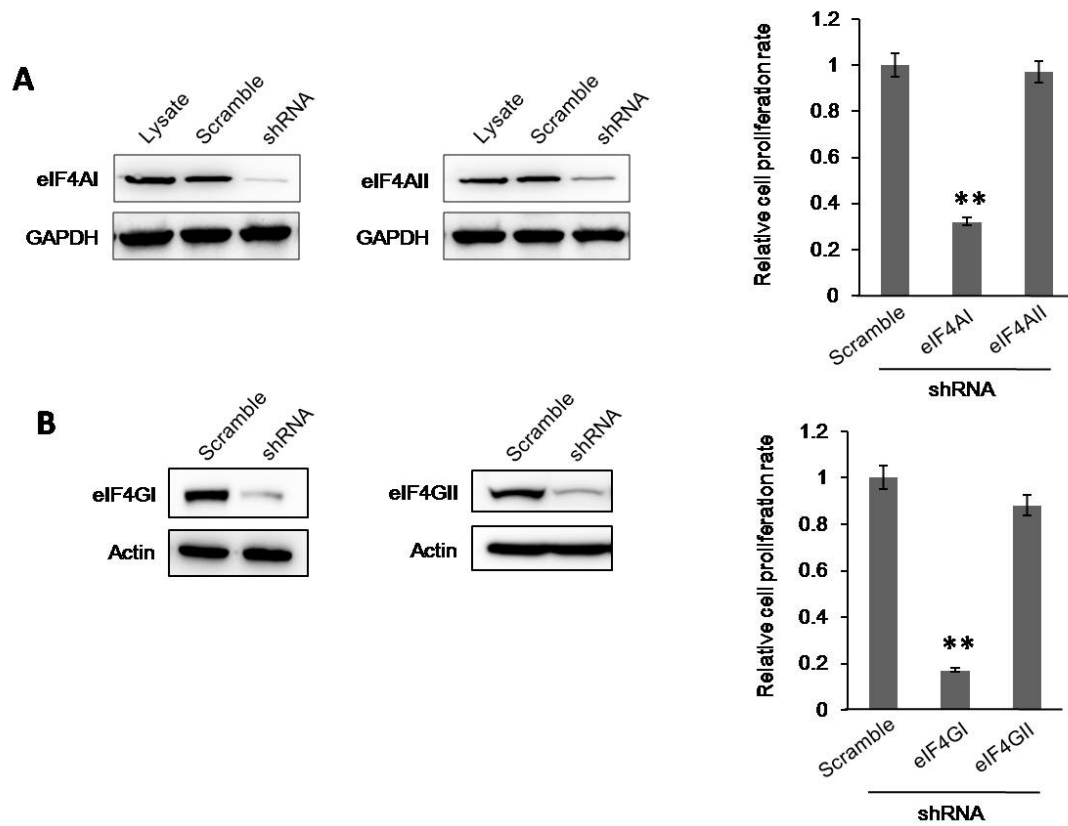


Figure S2. Selective depletion of isoforms eIF4AI (A) or eIF4GI (B) in 293T cells significantly impaired cell proliferation.

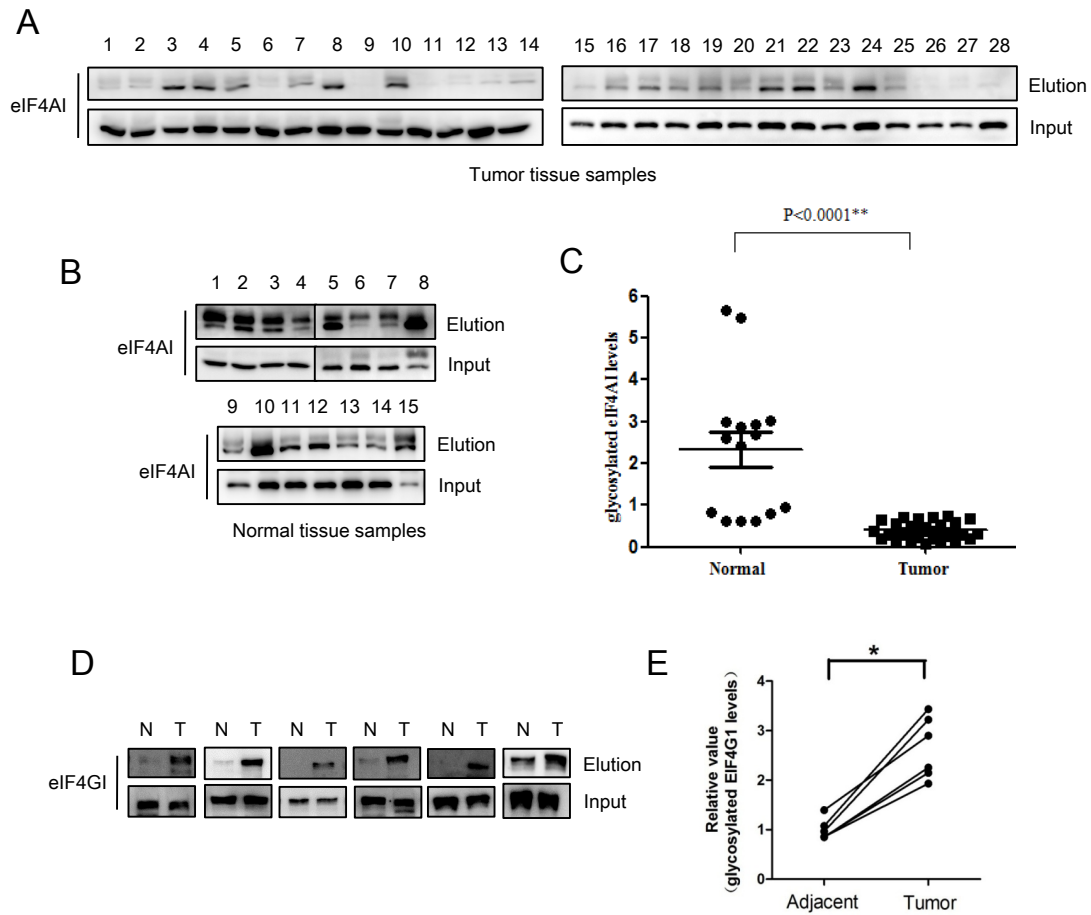


Figure S3. Analysis of eIF4A and eIF4G O-GlcNAcylation levels on human tumor tissue samples and normal tissue samples. (A) eIF4A glycosylation levels in human ovarian tumor tissue samples. (B) eIF4A glycosylation levels in human ovarian normal tissue samples. The levels of glycosylation were detected by the chemoenzymatic method and normalized with respect to eIF4AI protein levels. (C) Comparison and statistical analysis of eIF4A glycosylation levels between tumor and normal tissue samples. Error bars denote SEM. Statistical analysis was performed by Student's t test. (D) eIF4G glycosylation levels between human breast tumor tissue samples and the matching adjacent normal tissue samples. The levels of glycosylation were detected by the chemoenzymatic method and normalized with respect to eIF4G protein levels. (E) Comparison and statistical analysis of eIF4G glycosylation levels between tumor and normal tissue samples. Error bars denote SEM. Statistical analysis was performed by Student's t test.

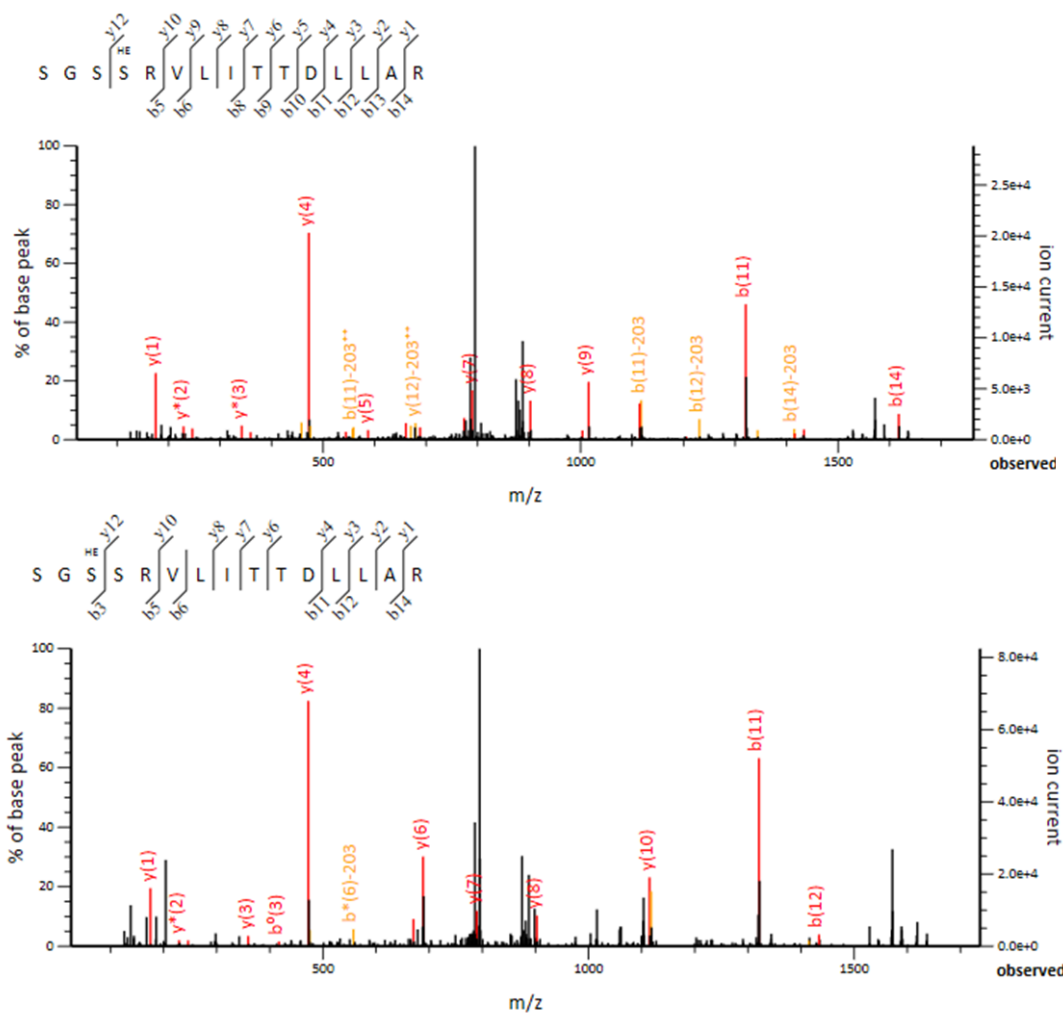


Figure S4. Mapping the sites of O-GlcNAcylation on eIF4AI using nanoLC-LTQ-CID/ETD-MS.

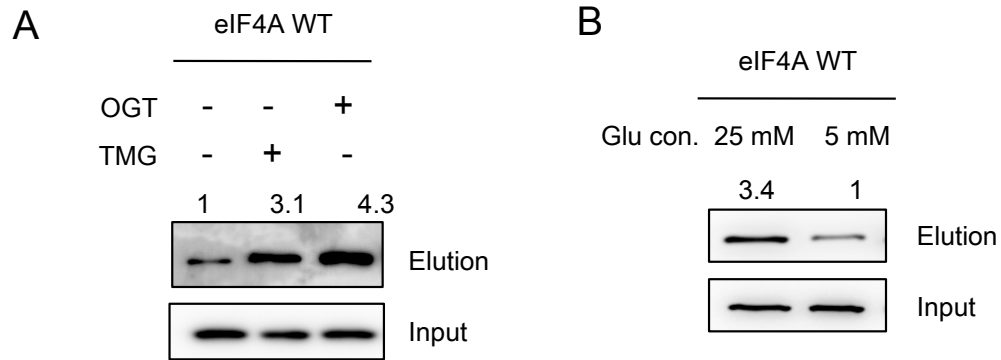


Figure S5. Quantification of fold change in eIF4A glycosylation levels upon cellular treatments. (A) Fold change in eIF4A glycosylation levels upon TMG treatment or OGT overexpression. The basal level of glycosylation (no treatment) was designated as 1. (B) Fold change in eIF4A glycosylation levels upon culturing with different concentrations of glucose. The basal level of glycosylation (5 mM glucose) was designated as 1.

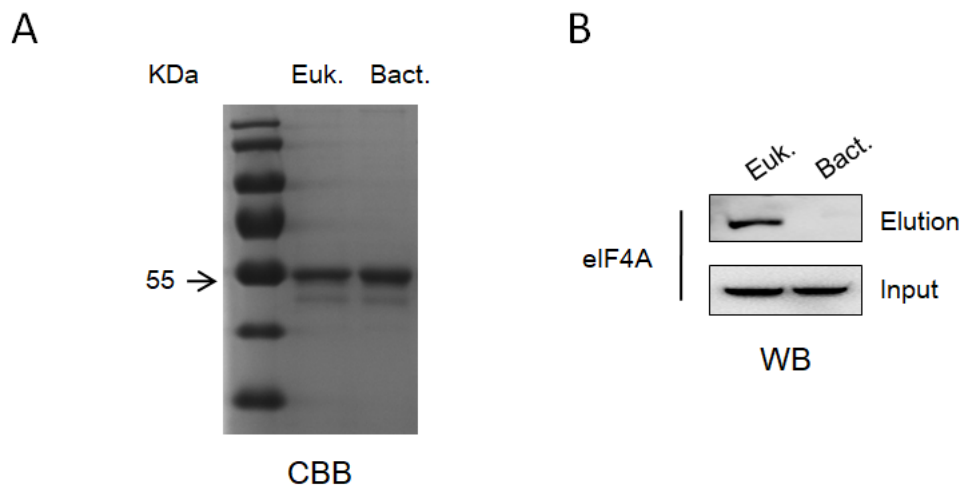


Figure S6. (A) Expression and purification of eIF4AI from 293T cells (Euk.) or *E. coli* BL21 (Bact.) host. (B) Verification of O-GlcNAcylation on eIF4AI from 293T cells.

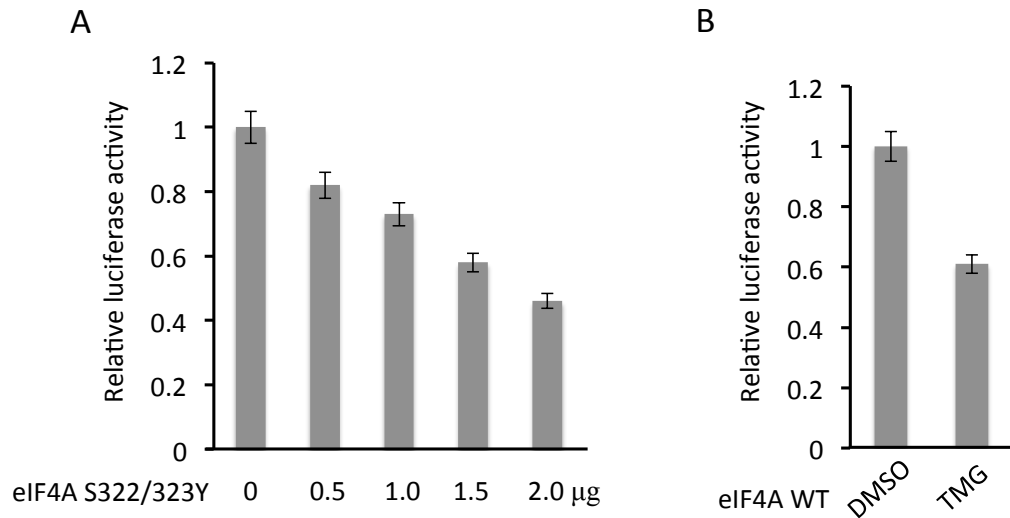


Figure S7. Measurement of in vitro translation activity in the presence of eIF4A mutant or glycosylated eIF4A. In vitro translation assays were performed using the Flexi® Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. (A) eIF4A S322/323Y mutant caused inhibition of in vitro translation in a dose-dependent manner. Different amounts of eIF4A S322/323Y mutant protein was added to the assay mixture in the presence of 1.0 µg eIF4A WT protein. (B) TMG-treated eIF4A caused inhibition of in vitro translation. 2.0 µg of the eIF4A protein was used in the assay. Triplicate experiments were performed.

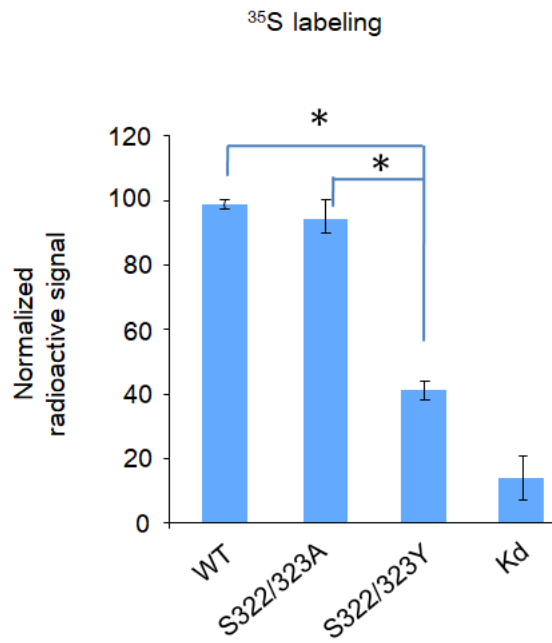


Figure S8. Determination of nascent protein synthesis in 293T cells expressing eIF4AI WT, S322/323A, and S322/323Y using [³⁵S]-methionine metabolic incorporation assays. The negative control was performed using cells with depletion of eIF4AI (Kd).

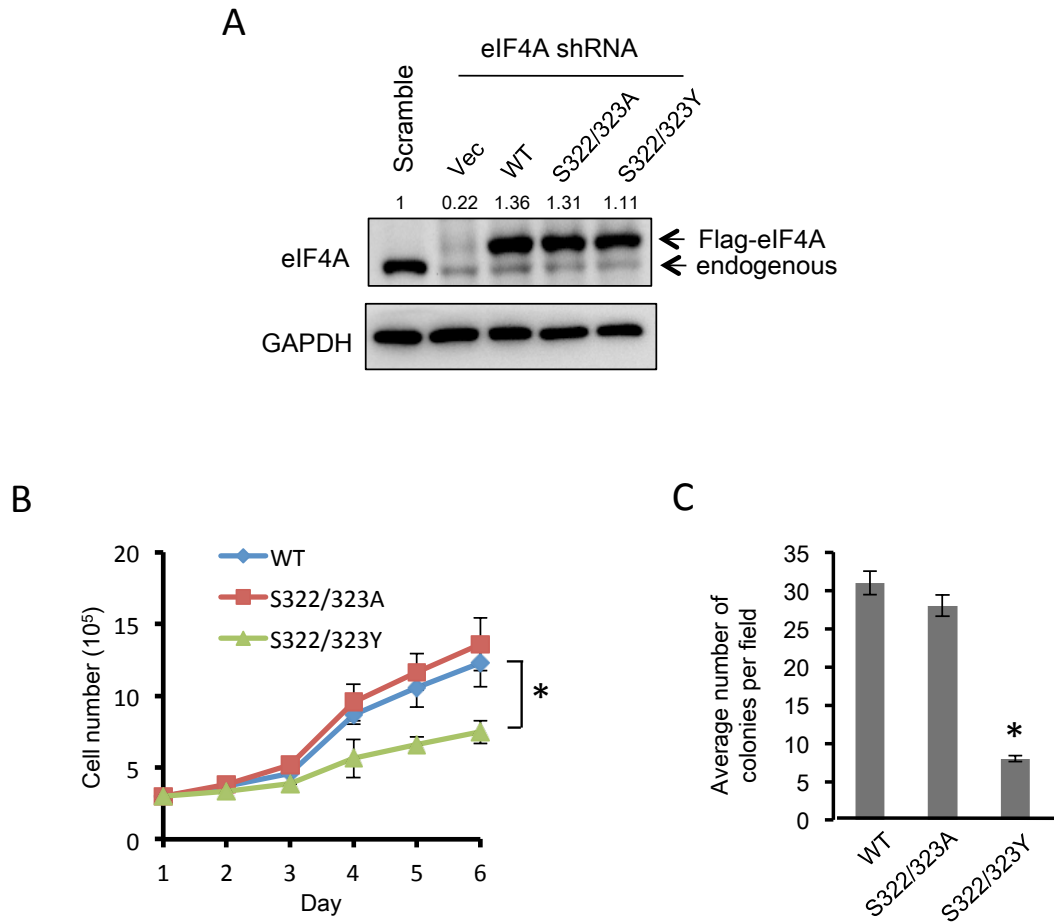


Figure S9. Glycosylation of eIF4AI negatively regulates cell proliferation and colony formation in vitro. (A) Generation of stable cell lines (A2780 ovarian cancer cells) that expressed Flag-tagged WT or mutant eIF4AI with simultaneous depletion of endogenous eIF4AI. (B) Determination of cell proliferation of A2780 cells stably expressing WT or mutant eIF4AI. (C) Determination of soft agar colony formation of A2780 cells stably expressing WT or mutant eIF4AI.

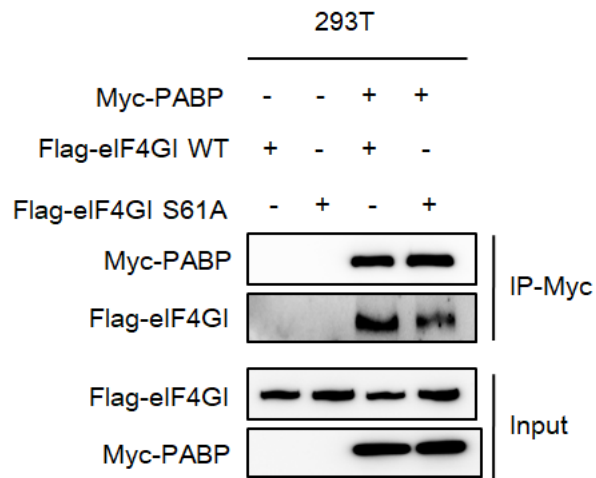


Figure S10. S61A eIF4GI mutant decreases the interaction with PABP. Flag-tagged WT, S61A eIF4GI, or Myc-tagged PABP was individually or co-expressed in 293T cells. Immunoprecipitation was carried out using anti-Myc antibodies, and immunoblotted with anti-Flag antibodies.

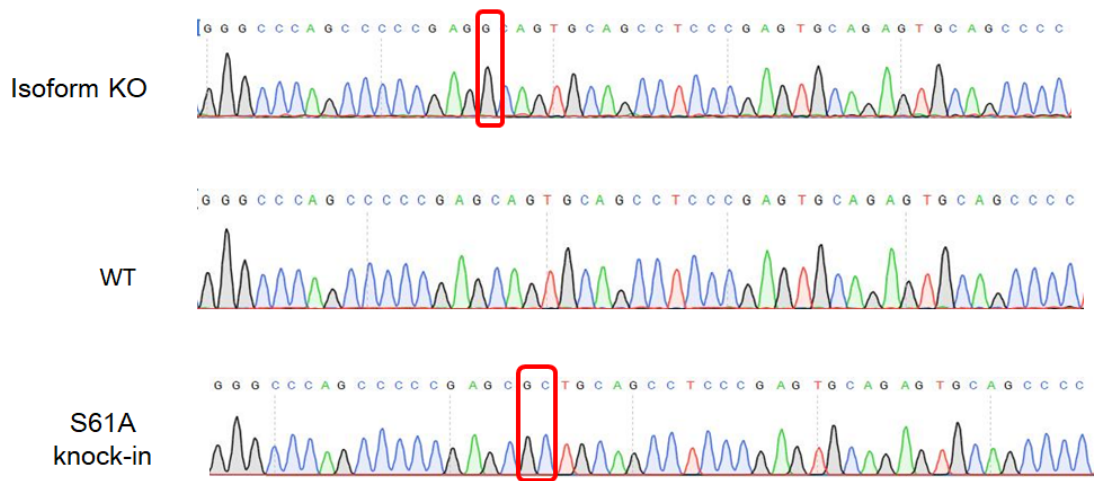


Figure S11. Verification of eIF4GI S61A knock-in and isoform-knockout (KO) MCF-7 cells by genomic sequencing.

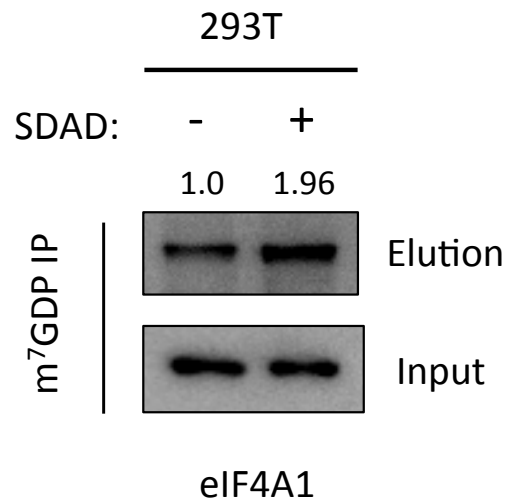


Figure S12. Analysis of eIF4A glycosylation levels in the eIF4F complex in the presence or absence of a chemical crosslinker. The compound Sulfo-NHS-SS-Diazirine was used to capture weak interactions in cell lysates. After crosslinking, the eIF4F complex was pulled down using m⁷GDP beads and eIF4A1 glycosylation levels were determined.

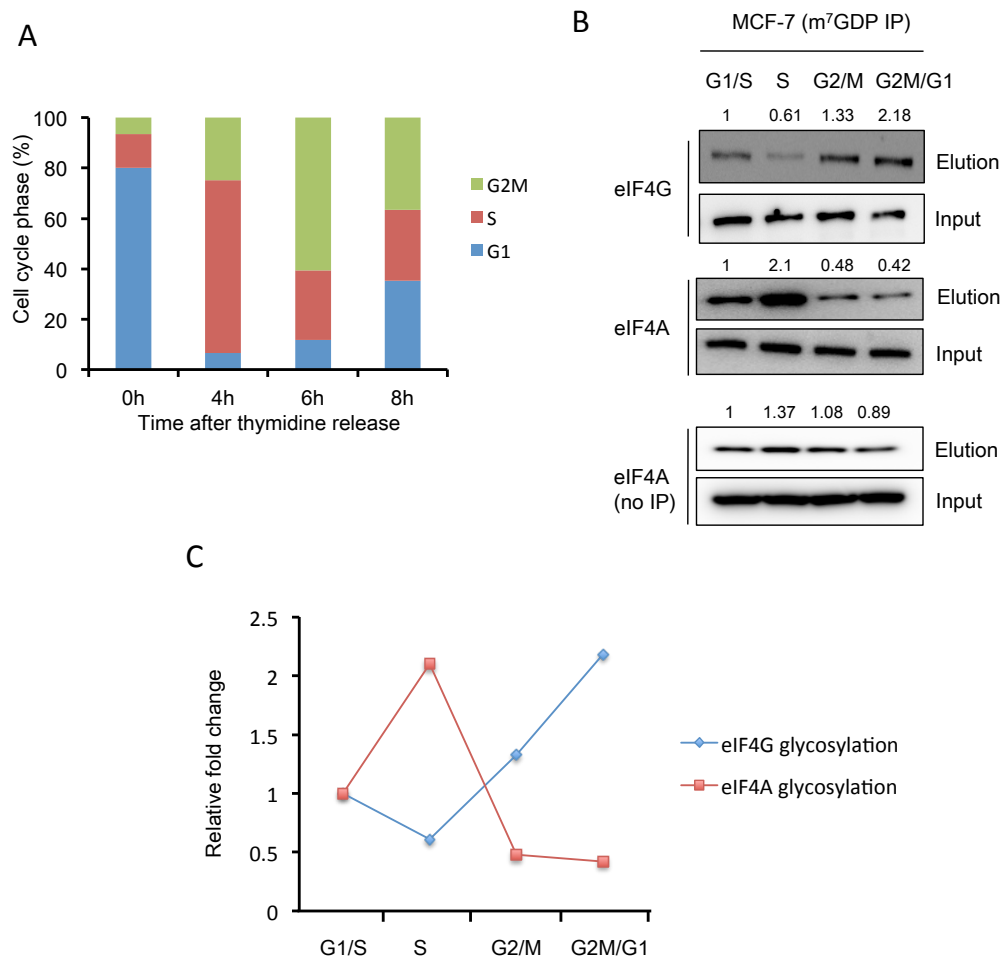


Figure S13. Determination of eIF4A and eIF4G glycosylation levels during the cell cycle progression. (A) Analysis of different cell cycle stages based on flow cytometry profiling of the DNA content of the cells. (B) Glycosylation levels of eIF4A and eIF4G at different cell cycle stages. (C) Quantification of fold change of glycosylation levels at different cell cycle stages.